Plant Cell Tiss Organ Cult (2014) 119:609–624
DOI 10.1007/s11240-014-0560-y

ORIGINAL PAPER

Isolation, phylogenetic relationship and expression profiling of sugar transporter genes in sweet orange (*Citrus sinensis*)

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Received: 18 January 2014 / Accepted: 15 June 2014 / Published online: 21 September 2014

Abstract Soluble sugars, including sucrose, glucose and fructose, are crucial components that determine the nutritional and commercial quality of sweet oranges (*Citrus sinensis*). Sugar transporters have been well demonstrated to mediate the long distance transportation and the subcelluar distribution of sugars in plants. Here, a genome-wide characterization of the sweet orange sugar transporter gene family was reported. We identified a set of putative orange sugar transporter genes containing 3 sucrose transporters (SUTs), 58 monosaccharide transporters (MSTs) that could be classified into 7 distinct subfamilies, and 16 SWEET transporters. Phylogenetic analysis among *Arabidopsis thaliana*, orange and *Vitis vinifera* allowed us to identify orthologous groups among these species. Three SUTs, *CsSUT1, CsSUT2* and *CsSUT4*, were expressed in fruits, and exhibited increased transcripts levels as fruit sucrose accumulated, which suggested they participated in fruit sucrose accumulation. A large number of MSTs, *CsSTP*s, *CsPMT*s, *CsVGT*s, *CspGlcT*s, *CsTMT*s, *CsERD6L*s and *CsSWEET*s showed fruit-expressed and up-regulated profiles, while glucose and fructose did not obviously accumulate as the fruit ripened. We then discussed the possibilities that fruit glucose and fructose had no evident accumulation, which was in contrast to sucrose. Additionally, many *cis*-elements such as ACGTATERD1, ARR1AT, MYCCONSENSUSAT, WRKY71OS, IBOXCORE, WBOXNTERF3, SUCROSE BOX 3 and WBOXHVISO1 were found in the promoter regions of orange sugar transporter genes, which suggested that they were transcriptionally regulated by sugars, phytohormones and stresses. This study might provide insights into the genomic organizations, evolutionary characteristics and expression profiling of the orange sugar transporter gene family.

Keywords Fructose · Glucose · Monosaccharide · Plasma membrane · Sucrose · Vacuole

Abbreviations

DAF	Days after flowering
ERD6L	Early response to dehydration 6-like
HT	Hexose transporter
INT	Inositol transporter
MST	Monosaccharide transporter
ORF	Open reading frame
PGlcT	Plastidic glucose transporter
PMT	Polyol/monosaccharide transporter
QRT-PCR	Quantitative reverse transcription-polymerase chain reaction
STP	Sugar transport protein
SUT	Sucrose uptake transporter
SWEET	Sugars will eventually be exported transporter
TMT	Tonoplast monosaccharide transporter
VGT	Vacuolar glucose transporter

Introduction

Soluble sugars not only serve as energy sources and skeleton molecules for plant growth and development, but also

Electronic supplementary material The online version of this article (doi:10.1007/s11240-014-0560-y) contains supplementary material, which is available to authorized users.

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functions as signal molecules to regulate the physiological activities and stress responses (Ramon et al. 2008). Soluble sugars are major nutritional components of fleshy fruits (Rai and Shekhawat 2013). In high plants, sugar transporters have been well demonstrated to play crucial roles in the long-distance transportation of sugars between source tissues and sink tissues via the phloem, and in the subcelluar distribution among various tissues (Kuhn and Grof 2010). Currently, most studied sugar transporters, which present a classic structure with 12 putative transmembrane helices and 11 loops, belong to the Major Facilitator Superfamily (MFS) (Shiratake 2007). Until now, sugar transporters mainly sucrose transporters (SUTs) and monosaccharide transporters (MSTs) have been extensively isolated from many herbaceous plants including *Arabidopsis thaliana* (Buttner 2007; Sivitz et al. 2008; Wormit et al. 2006), *Fragaria* × *ananassa* (Jia et al. 2013), *Medicago truncatula* (Doidy et al. 2012), *Nicotiana tabacum* (Okubo-Kurihara et al. 2011) and *Solanum lycopersicum* (McCurdy et al. 2010; Hackel et al. 2006), and from woody plants such as *Populus tremula* × *alba* (Payyavula et al. 2011) and *Rosa hybrida* (Henry et al. 2011). However, they have been isolated from few perennial woody fruit trees besides *Juglans regia* (Decourteix et al. 2006, 2008), 'Murcott' tangerine (*C. reticulate* × *C. sinensis*) (Li et al. 2003), *Malus domestica* (Li et al. 2012) and *Vitis vinifera* (Afoufa-Bastien et al. 2010). Recently, a novel sugar transporter SWEET (Sugars Will Eventually Be Exported Transporter) family is characterized as being involved in sugar partition, and isolated from *Arabidopsis* and *Oryza sativa* (Chardon et al. 2013; Chen et al. 2010, 2012; Guo et al. 2013; Klemens et al. 2013).

Genome sequencing has revealed that *SUT*s are encoded by a rather small multigenic family with nine members in *Arabidopsis* (Sauer et al. 2004; Sauer 2007), four in grape (Afoufa-Bastien et al. 2010) and five in apple (Li et al. 2012). To date, most cloned SUTs have been identified as sucrose proton symporters (Kuhn and Grof 2010), except sucrose facilitators from *Pisum sativum* and *Phaseolus vulgaris* (Zhou et al. 2007). A phylogenetic comparison reveals that all known SUTs could be divided into five clades (Ayre 2011; Kuhn and Grof 2010). Dicotyledons possess only three clades SUT1, 2 and 4, and the SUT3 and SUT5 clades are specific to monocotyledons. The SUT1 clade plays roles in phloem loading in source tissues and sucrose uptake in sink tissues (Hackel et al. 2006; Sivitz et al. 2008). The SUT2 clade possesses an extended N-terminus and a central loop (Barker et al. 2000). The SUT4 clade members are tonoplast-localized sucrose/proton symporters that regulate sucrose distribution between vacuoles and the cytosol in leaves and fruits (Eom et al. 2011; Jia et al. 2013; Payyavula et al. 2011; Schneider et al. 2012).

*MST*s, containing seven distinct subfamilies such as sugar transport protein, early response to dehydration 6-like (ERD6L) and tonoplast monosaccharide transporter, are encoded by a far more diverse multigenic family in plants (Buttner 2007). For example, there are 53 and 59 members in *Arabidopsis* and grape, respectively (Afoufa-Bastien et al. 2010; Buttner 2007). Sugar transport proteins (STPs, also known as hexose transporters, HTs), which are located in the plasma membrane, are identified as monosaccharides proton symporters and play roles in monosaccharides import into sink cells (Buttner 2010). Most *STP* members are sink-specific and responsible for sink development (Buttner 2007). In fruits, related studies demonstrate that *STP*s play important roles in sugar accumulation. For example, the RNAi-mediated knockdown of three hexose transporters in tomato show an obvious decrease of hexoses content in fruits, which confirm that they mediate the transportation of hexoses into tomato fruit cells (McCurdy et al. 2010). *VvHT1*, *VvHT2* and *VvHT3* are expressed at different stages of grape berry development, which suggest that the various members of the *VvHT*s family are involved in berry development (Hayes et al. 2007; Vignault et al. 2005). In addition, other MSTs subfamilies are identified as tonoplast-located MSTs. *Arabidopsis* AtVGT1 (vacuolar glucose transporter) and AtTMTs (tonoplast monosaccharide transporter) as well as rice OsTMTs are found to import hexoses into the vacuole and to play important roles in vacuole sugar partition (Aluri and Buttner 2007; Cho et al. 2010; Schulz et al. 2011; Wingenter et al. 2010; Wormit et al. 2006). Then, AtERDL6, which belongs to the ERD6L subfamily, is reported to function as a tonoplast-localized glucose exporter and release glucose from vacuoles into the cytosol (Poschet et al. 2011).

Citrus fruit ripening is accompanied by the high accumulation of soluble sugars. Fruit sugar accumulation is well known to be mainly determined by three processes: sugar transport, metabolism, and storage (Katz et al. 2007). Over the past decades, a series of studies have intensively demonstrated that the metabolism-related enzymes of sugars affect the sugar accumulation of citrus fruits (Katz et al. 2011; Komatsu et al. 2002). Meanwhile, there have been many studies on sugar transport in citrus fruit (Etxeberria et al. 2005; Huberman et al. 2005; Koch and Avigne 1990). However, a comprehensive study on the sugar transport processes in citrus fruit is still needed. The putative sugar transporters participating in sugar transport processes and their expression profiles, regulation mechanism and functions are still unknown.

In recent years, within the grape and apple genome sequences, a series of putative genes encoding sugar transporters have been isolated, and the main candidate genes functioning in various tissues and in fruit sugar accumulation have been identified by expression profiles analysis (Afoufa-Bastien et al. 2010; Li et al. 2012). The

release of the sweet orange genome sequences has provided us with a convenient opportunity to study the candidate genes contributing to fruit sugar accumulation (Xu et al. 2013). In the present study, a genome-wide characterization of putative genes encoding orange sugar transporters is performed. Firstly, the putative orange sugar transporter gene family is searched, and the phylogenetic analysis with corresponding genes from *Arabidopsis*, grape and other species is conducted. Then, the gene expression patterns in the source tissue (leaf) and sink tissues (flower, fruit and callus), and the gene expression patterns during fruit development are profiled. Finally, the *cis*-elements in promoter regions are searched and summarized.

Materials and methods

Fruits collection

The 'Anliu' sweet orange (*Citrus sinensis* L. Osbeck), grown in the Institute of Citrus Research in Guilin City, Guangxi Province, China, was used as experimental material in this study. The fruit samples were harvested at 120, 150, 180 and 210 DAF (days after flowering) from five separate trees which were at the same age and growth conditions. At each sampling stage, a total of 20 fruits from five trees were harvested. Fruits were immediately separated into peel and pulp. The pulp was rapidly frozen in liquid nitrogen and kept at −80 °C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted according to the methods of Liu et al. (2006). The quality of total RNA was detected by electrophoresis in agarose gel, and the concentrations were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Total RNA samples were digested with DNase I to avoid genomic DNA contamination. Then, cDNA was synthesized with 1.5 μg of RNA samples according to the manufacturer's instructions of RevertAid First Strand cDNA synthesis kit (Fermentas, Lithuania).

Measurement of sugar contents in fruits

Content of soluble sugars was measured by the gas chromatography (Yu et al. 2012). Three independent replicates were employed for each sample analysis.

Candidate genes identification

The candidate genes encoding sweet orange sugar transporters were retrieved by BLASTP searching against the sweet orange genome (http://citrus.hzau.edu.cn/orange/), using *Arabidopsis* sugar transporter proteins as queries with E-value of 1, 00^{E-05} as threshold. The *Arabidopsis* sugar transporter proteins contained sucrose transporter family, monosaccharide transporter family and SWEET transporter family. For each candidate gene, the 2,000 bp upstream sequence of the start codon was downloaded to be promoter sequence.

Sequence analysis and phylogenetic comparison

Sequence similarities of the deduced amino acid were calculated with the Clustal V multiple alignments in Lasergene software (DNASTAR, USA). Sequences alignment was performed using the MUSCLE program (Edgar 2004), and the maximum likelihood (ML) analysis was carried out with the PHYML program (http://www.phylogeny.fr/) and the JTT amino acid substitution model (Dereeper et al. 2008). To test the reliability of the phylogeny trees, 100 replicates were calculated for bootstrap analysis (Anisimova and Gascuel 2006; Guindon and Gascuel 2003). The phylogenetic tree was visualized with Treedyn program (Chevenet et al. 2006). MapChart was employed to display the chromosomal location of isolated genes (Voorrips 2002). The *cis*-elements in promoter sequences were searched using the Plant Cis acting regulatory DNA Elements database (http://www.dna.affrc.go.jp/PLACE/index.html) (Higo et al. 1999; Prestridge 1991).

Transcription profile among different tissues and during fruit development

The normalized expression values (RPKM) in several tissues were retrieved from the RNA-sequencing database (Xu et al. 2013). Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was carried out to detect the transcription profiles during fruit development. The gene-specific primers were designed with Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). The orange *Actin* gene was used for reference gene (Liu et al. 2007). Specificity of each primer pair was confirmed by BLASTN searching in the orange genome database. The primer sequences of target genes and reference gene were presented in Table S2. QRT-PCR was performed on the ABI 7900HT Fast Real Time System (PE Applied Biosystems, Foster City, CA, USA) using the SYBR-Green PCR Master Mix (Applied Biosystems). Reactions program were performed as follows: 50 °C/120 s, 95 °C/60 s, and then cycled at 95 °C/15 s and 60 °C/60 s for 40 cycles. The relative expression values were calculated with the $2^{-\Delta\Delta CT}$ method.

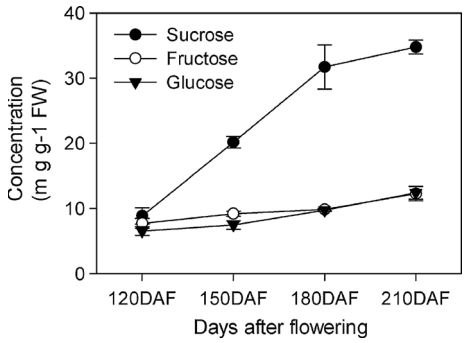

Fig. 1 Concentration of soluble sugars (sucrose, glucose and fructose) during sweet orange (*C. sinensis*) fruit development. Values were mean ± standard error of three replicates

Results

Soluble sugars content during orange fruit development

The soluble sugars content during orange fruit development was detected by a GC (gas chromatography) approach. The results were shown in Fig. 1. At 120DAF, the content of sucrose, fructose and glucose was almost equal. Then, the fructose and glucose content increased slightly during fruit development. The rate of sucrose accumulation was rapid before 180DAF, and then slowed down. The sucrose content accounted for more than 60 % of the total soluble sugars at the fruit ripening.

Chromosomal location and phylogenetic relationship of the orange sugar transporter genes

To isolate putative genes encoding the sugar transporters, a BLASTP searching against the sweet orange genome was performed using *Arabidopsis* sugar transporter proteins as queries. In total, 77 genes encoding 3 SUTs, 58 MSTs and 16 SWEET transporters were isolated (Table S1). The chromosomal locations were predicted. As shown in Fig. S1, approximately 50 % (34/71) of the putative genes were located on the chromosomes 3 and 9. Then, a phylogenetic tree of the 3 SUTs and 58 MSTs was constructed with protein sequences using the ML method. The result showed that they could be classified into 8 distinct subfamilies (Fig. S2), and the detailed descriptions were given below.

Citrus sinensis Sucrose Transporter (*CsSUT*) family

Three ORFs showed 38.6–71.5 % similarity to the *Arabidopsis* SUC members and 38.8–76.0 % similarity to the grape SUT members. CsSUT1 had 46.5 % similarity to CsSUT4, and 38.6 % similarity to CsSUT2. *CsSUT1* and *CsSUT2* were identical to *CitSUT1* and *CitSUT2*, which were reported in a previous research (Li et al. 2003). Transmembrane domains prediction suggested that all three CsSUTs had 12 transmembrane helices and 11 loops (data not shown). It showed that *CsSUT1* and *CsSUT4* both had 4 exons, and that *CsSUT2* had 14. A phylogenetic analysis with SUT members from *Arabidopsis*, apple, grape and rice showed that three major clades were obviously separated, as supported by bootstrap values of 100 %. The three CsSUTs were located in the SUT1, SUT2 and SUT4 clade, respectively (Fig. 2a).

Citrus sinensis Sugar Transport Protein (*CsSTP*) subfamily

Twenty-five ORFs displayed 29.4–80.7 % similarity to the *Arabidopsis* STP subfamily and 10.3–83.2 % similarity to the grape HT subfamily. There was also 19.5–90.3 % similarity among the 25 ORFs. Among all the CsSTPs, Cs9g15170 was much longer than the other STPs, which was found to represent two fussed STPs. The CsSTP5, CsSTP17, CsSTP19 and CsSTP25 sequences were obviously shorter than the other MSTs. CsSTP16, CsSTP18 and CsSTP20 lacked approximately 50 amino acids in the N-terminus. A comparison of the chromosome distribution revealed that almost 60 % (14/25) of the members were located on chromosome 9, forming two tandem repeat regions: *CsSTP6*, *8* and *13*, and *CsSTP11*, *12* and *15–24* (Fig. S1). A phylogenetic analysis of *Arabidopsis*, grape and orange STP subfamily was displayed in Fig. 3a. Four orthologous groups among the three species were identified, i.e., AtSTP13/CsSTP13/VvHT5, AtSTP7/CsSTP7/VvHT3, AtSTP14/CsSTP14/VvHT13 and AtSTP5/CsSTP10/VvHT2, which were supported by bootstrap values of 100 %. AtSTP3, VvHT4 and the genes in the tandem repeat region containing CsSTP11, 12, 15–25, were located together. CsSTP8 with grape VvHT12 and the grape tandem repeat genes cluster VvHT14–24 were located together.

We further investigated *CsSTP11*, *12* and *15–24*, which represented the largest tandem repeat cluster in the *CsSTP*s subfamily. We investigated the neighboring genes in *Arabidopsis*, grape and orange. On the one side of *AtSTP3* (At5g61520), an *ADH* gene (alcohol dehydrogenase family protein, At5g61510) of *Arabidopsis* was found to be an ortholog of the *ADH* gene (Cs9g15140) in orange (Fig. 4); on the other side of *AtSTP3*, the *Rho GTPase* gene (Rho GTPase-activating protein, At5g61530) and *Ntn hydrolases* gene (N-terminal nucleophile aminohydrolases, At5g61540) of *Arabidopsis* were orthologs of *Rho GTPase* (Cs9g15310) and *Ntn hydrolases* (Cs9g15320) of orange, respectively. *CsSTP11*, *12* and *15–24* were tandemly arranged in the region between the *ADH* and *Rho GTPase* gene of orange on chromosome 9. Such an orthologous relationship was not found in the regions adjacent to grape *VvHT4*.

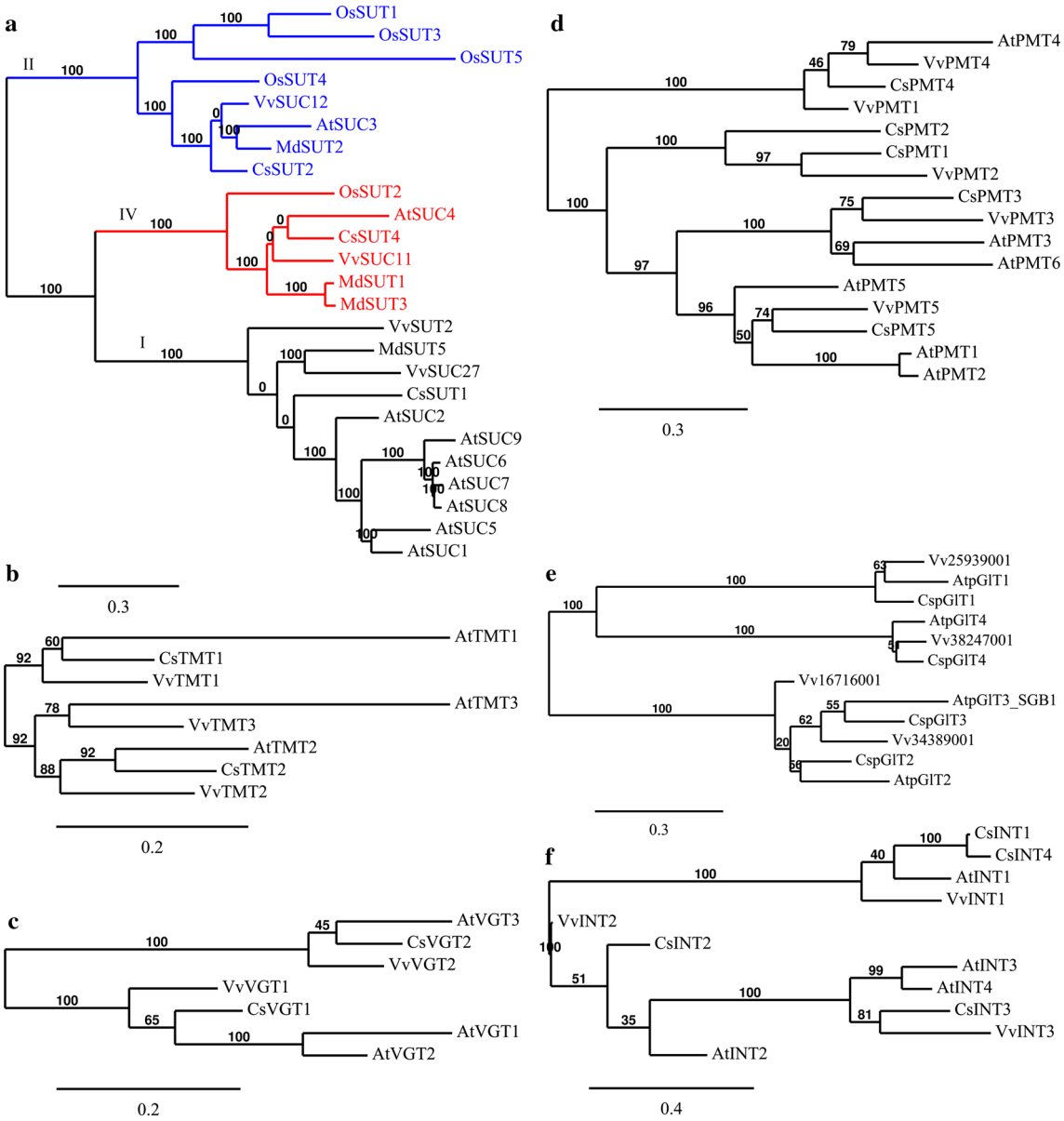

Fig. 2 Maximum likelihood phylogeny of SUTs and MSTs among *C. sinensis* and other species. (**a**) SUTs, (**b**) TMTs, (**c**) VGTs, (**d**) PMTs, (**e**) pGlcTs and (**f**) INTs. The trees were constructed by MUSCLE and PhyML with the JTT amino acid substitution model. Bootstrap values were analyzed with 100 replicates. Accession numbers were: *A. thaliana*: AtSUC1–9 (At1g71880, At1g22710, At2g02860, At1g09960, At1g71890, At5g43610, At1g66570, At2g14670 and At5g06170), AtTMT1–3 (At1g20840, At4g35300 and At3g51490), AtVGT1–3 (At3g03090, At5g17010 and At5g59250), AtpGlcT1–4 (At5g16150, At1g67300, At1g79820 and At1g05030), AtINT1–4 (At2g43330, At1g30220, At2g35740 and At4g16480) and AtPMT1–6 (At2g16120, At2g16130, At2g18480, At2g20780, At3g18830 and At4g36670); *M. domestica*: MdSUT1–5 (Li et al. 2012); *Oryza sativa*: OsSUT1–5 (AAF90181, BAC67163, BAB68368, BAC67164 and BAC67165); *V. vinifera*: VvSUC11 (AF021808), VvSUC12 (AF021809), VvSUC27 (AF021810) and VvSUT2 (ADP37124), VvVGTs, VvTMTs, VvPMTs, VvpGlcTs and VvINTs (Afoufa-Bastien et al. 2010); *C. sinensis* (Table S1)

Citrus sinensis Early Response to Dehydration 6-like (*CsERD6L*) subfamily

Sixteen ORFs had 25.4–77.2 % similarity to the *Arabidopsis* ERD6L subfamily and 15.7–81.9 % similarity to the grape ERD6L subfamily. There was also 19.5–90.3 % similarity among the 16 ORFs. The chromosomal location patterns showed that nine and three ORFs were located on chromosomes 3 and 5, respectively, forming two tandem repeat regions (Fig. S1). A phylogenetic analysis of *Arabidopsis*, grape and orange ERD6L subfamily was shown in Fig. 3b. There were two orthologous groups

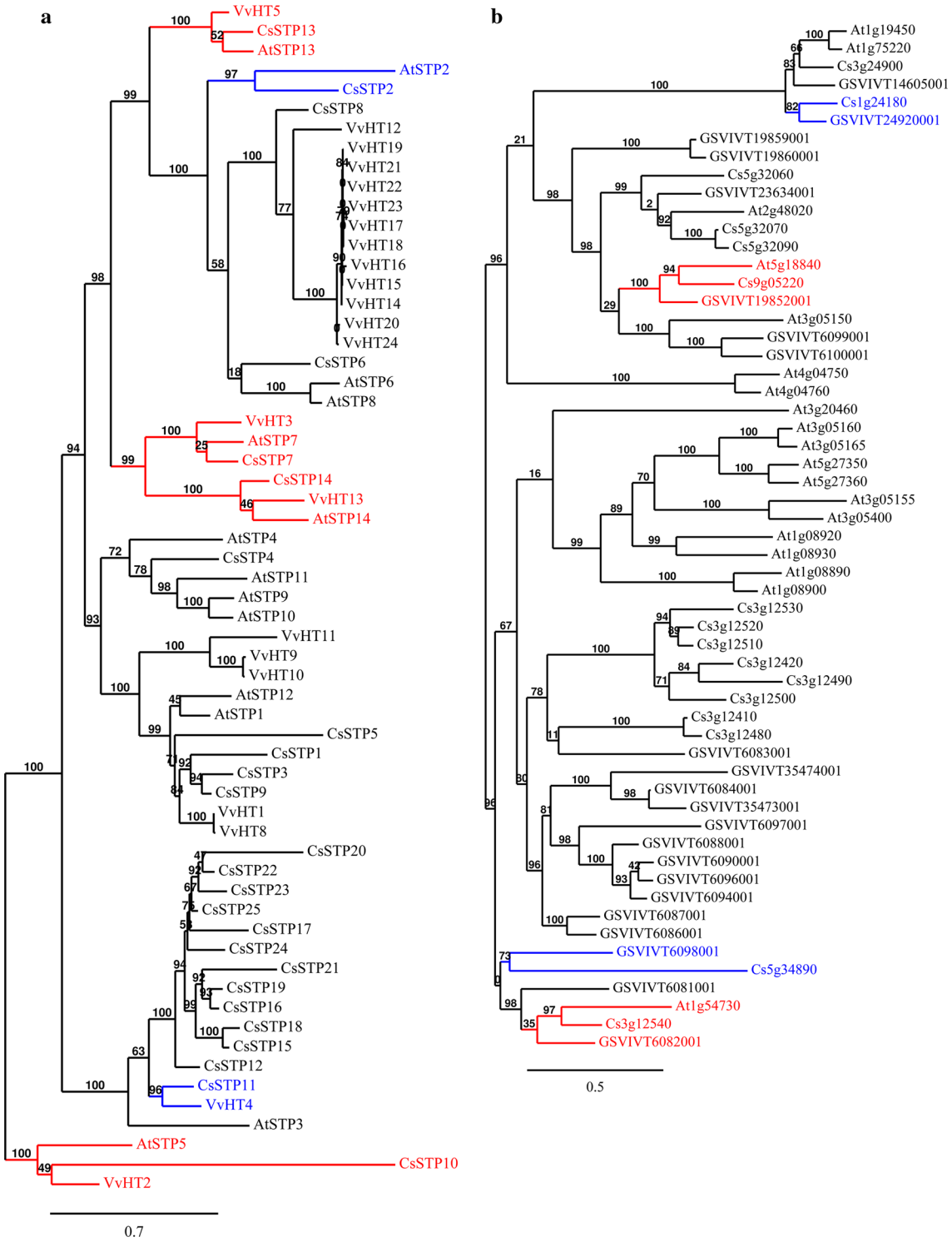

Fig. 3 Maximum likelihood phylogeny of STPs (**a**) and ERD6Ls (**b**) among *A. thaliana, C. sinensis* and *V. vinifera.* The trees were constructed by MUSCLE and PhyML with the JTT amino acid substitution model, a discrete gamma model with 4 categories and estimated shape parameters of 0.98 and 1.322, respectively. Bootstrapping was calculated with 100 replicates. Accession numbers were: *A. thaliana* AtSTP1–14 (At1g11260, At1g07340, At5g61520, At3g19930, At1g34580, At3g05960, At4g02050, At5g26250, At1g50310, At3g19940, At5g23270, At4g21480, At5g26340 and At1g77210), *V. vinifera* HTs and ERD6Ls (Afoufa-Bastien et al. 2010), and *C. sinensis* STPs and ERD6Ls (Table S1)

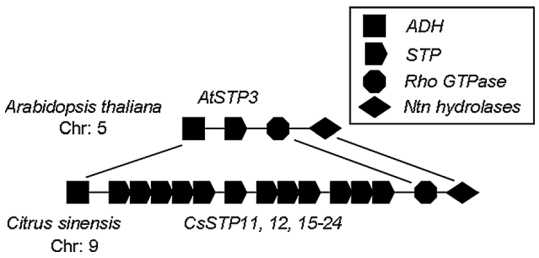

Fig. 4 The collinearity of chromosomal segments containing the tandem repeat cluster *CsSTP11*, *12* and *15–24* on orange chromosome 9 and *AtSTP3* on *Arabidopsis* chromosome 5. The adjacent genes were arranged as follows in *Arabidopsis*: *ADH* (alcohol dehydrogenase family protein, At5g61510), *AtSTP3* (At5g61520), *Rho GTPase* (Rho GTPase-activating protein, At5g61530) and *Ntn hydrolases* (N-terminal nucleophile aminohydrolases, At5g61540); the adjacent orthologous genes were arranged as follows in *C. sinensis*: *ADH* (Cs9g15140), *CsSTP11*, *12* and *15–24* (Table S1), *Rho GTPase* (Cs9g15310) and *Ntn hydrolases* (Cs9g15320)

among *Arabidopsis*, grape and orange: At5g18840, Cs9g05220 and GSVIVT19852001; At1g54730, Cs3g12540 and GSVIVT6082001. Two orthologous pairs GSVIVT24920001 and Cs1g24180, GSVIVT60980001 and Cs5g34890 were found between grape and orange. GSVIVT6083001 from the grape ERD6L subfamily together with eight orange ERD6L members formed a single clade. Two large groups contained only *Arabidopsis* and grape ERD6L members.

Citrus sinensis Tonoplast Monosaccharide Transporter (*CsTMT*) and Vacuolar Glucose Transporter (*CsVGT*) subfamilies

Two ORFs had 50.5–76 % similarity to the *Arabidopsis* AtTMT1–3 and 73.7–80.5 % similarity to the grape VvTMT1–3. There was 72.4 % similarity between the two ORFs. The exon/intron organizations showed that they both had five exons and four introns. A phylogenetic analysis displayed that two distinct clades were divided (Fig. 2b). CsTMT1 had high homology to AtTMT1 and VvTMT1. CsTMT2 together with VvTMT2 and AtTMT2 belonged to the other clade. Orange lacked the *Arabidopsis* or grape TMT3-like isoforms.

Two ORFs presented 55.3–76.1 % similarity to the *Arabidopsis* AtVGT1–3 and 59.3–83.2 % similarity to the grape VvVGT1–2. There was 59.5 % similarity between the two ORFs. *CsVGT1* and *CsVGT2* both had 15 exons and 14 introns. A phylogenetic analysis showed that two distinct clades were separated, with supporting by the bootstrap values of 100 % (Fig. 2c). CsVGT1, VvVGT1 and AtVGT1, 2 were located in a single clade. CsVGT2, VvVGT2 and AtVGT3 formed the other clade.

Citrus sinensis Plastidic Glucose Transporter (*CspGlcT*) subfamily

Four ORFs had 37.8–78.1 % similarity to the AtpGlcT1–4 and 35.8–80.3 % similarity to the grape VvpGlcT1–4. There was 37.8–70.6 % similarity among the four ORFs. The four *CspGlcT* members had 13 exons and 12 introns, except *CspGlcT2*, which had 12 exons and 11 introns. A phylogenetic analysis showed that the pGlcT subfamily was divided into three clades, as supported by bootstrap values of 100 % (Fig. 2e). CspGlcT2, CspGlTc3, AtpGlcT2, AtpGlcT3/AtSGB1, Vv16716001 and Vv34389001 from grape were located in a separate clade. CspGlcT1, AtpGlcT1 and Vv25939001; CspGlcT4, AtpGlcT4 and Vv38247001 were located in two other clades, respectively.

Citrus sinensis Inositol Transporter (*CsINT*) and Polyol/Monosaccharide Transporter (*CsPMT*) subfamilies

Five ORFs had 42–71 % similarity to the *Arabidopsis* AtPMT1–6 and 41.6–77.2 % similarity to the grape VvPMT1–5. There was 43.6–96.8 % similarity among the five ORFs. The exon/intron organizations showed that they had six or five exons. A phylogenetic analysis showed that four distinct clades were separated (Fig. 2d). The three clades were divided as follows: AtPMT4, CsPMT4 and VvPMT1, 4; AtPMT3, 6, CsPMT3 and VvPMT3; AtPMT1, 2, 5, CsPMT5 and VvPMT5. The three clades contained *Arabidopsis*, grape and orange polyol/monosaccharide transporter (PMT) members. CsPMT1, 2 and VvPMT2 formed a single clade, which contained only grape and orange PMT members.

Four ORFs had 35.6–81 % similarity to the *Arabidopsis* AtINT1–4 and 42.4–83.5 % similarity to the grape VvINT1–3. There was 40.7–91.5 % similarity among the four ORFs. CsINT1 was obviously shorter than the other three members. CsINT2–4 had five or four exons, and CsINT1 had two. A phylogenetic analysis showed that two distinct clades were divided. CsINT1 and 4, AtINT1 and VvINT1 were located in a single clade (Fig. 2f).

Citrus sinensis SWEET transporter (*CsSWEET*) family

Sixteen ORFs showed 18.8–72.5 % similarity to the *Arabidopsis* AtSWEET1–17. There was 20.4–62.6 % similarity among the 16 ORFs. The chromosomal locations of two members (orange1.1t02626 and orange1.1t02627) were not identified. Most *CsSWEET*s had five or six exons. A phylogenetic analysis with *Arabidopsis* AtSWEET1–17 showed that four clades were divided, as supported by bootstrap values of more than 85 % (Fig. 5). Cs2g04140, Cs3g20720, Cs5g13290, Cs9g18460, and AtSWEET1–3

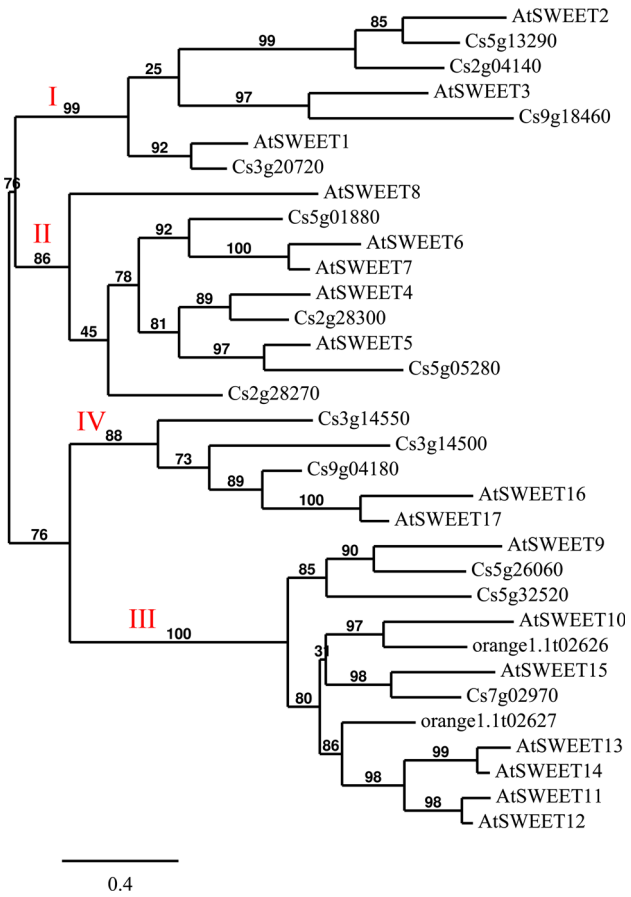

Fig. 5 Maximum likelihood phylogeny of the SWEET transporters among *A. thaliana* and *C. sinensis*. The tree was constructed by MUSCLE and PhyML with the JTT amino acid substitution model, a discrete gamma model with 4 categories and an estimated shape parameter of 1.252. Bootstrapping was calculated with 100 replicates. *C. sinensis* SWEETs (Table S1)

belonged to the clade I. Cs2g28270, Cs2g28300, Cs5g01880, Cs5g05280 and AtSWEET4–8 were grouped into the clade II. Cs3g14500, Cs3g14550, Cs9g04180 and AtSWEET16–17 formed the clade IV.

Expression patterns of sugar transporter genes in various orange tissues

The expression patterns of the sugar transporter genes between the source and sink tissues suggested their physiological roles. To survey the expression patterns of the sugar transporter genes among different tissues, we analyzed the digital expression normalized (RPKM) data from our RNA-sequencing project among leaves, flowers, fruits and calluses (Xu et al. 2013).

As shown in Fig. 6a, *CsSUT1*, *CsSUT2* and *CsSUT4* all showed relative expression levels in leaves, flowers, calluses and fruits, and *CsSUT4* showed high expression in fruits. *CsVGT1–2* and *CsTMT1–2* were detected in all tested tissues, and *CsTMT2* had higher expression in fruits and calluses than in flowers and leaves. *CspGlcT1–4* were expressed in all tested tissues. *CsPMT1* was highly expressed in flowers. *CsPMT3* and *CsINT4* showed preferential expression levels in calluses. The *CsPMT4*, *5* and *CsINT1* transcripts were abundant in flowers, fruits and leaves. *CsPMT2* was rare in leaves. *CsINT2, 3* were expressed in all tested tissues, and *CsINT3* had the highest expression level in leaves.

For the orange *STP* subfamily (Fig. 6b), approximately two thirds (15/23) of the *STP*s were weakly detected in tissues. *CsSTP4*, *6* and *8* were abundant in calluses, and weak in other tissues. *CsSTP1*, *7*, *13* and *14* were strongly expressed in all tested tissues. The *CsSTP11* transcripts were more detected in leaves and fruits. *CsSTP5*, *22*, *23* and *25* displayed significantly high expression levels in calluses and fruits. *CsSTP16*, *19* and *21* were preferentially expressed in leaves. *CsSTP2*, *9* and *24* were highly expressed in flowers. *CsSTP3*, *10*, *17*, *18* and *20* were rarely detected in all tested tissues. In total, there were 9 *CsSTP*s that showed significant expression levels in fruits.

For the orange *ERD6L* subfamily (Fig. 6c), Cs3g12410, Cs5g34890 and Cs3g12480 were weakly expressed in all tested tissues, except Cs3g12410, which was expressed in leaves. Cs3g12420 was preferentially expressed in calluses. The other 12 *CsERD6Ls* were expressed in all tissues. The Cs5g32070 and Cs5g32090 transcripts were detected in all tested tissues and highly expressed in fruits. Cs3g12490 and Cs3g12500 were strongly detected in calluses than in leaves, flowers and fruits. The Cs3g12510 and Cs3g12540 transcripts were more abundant in leaves and flowers. Cs9g055220 was more abundant in sink tissues fruits, calluses and flowers. In total, there were 12 *CsERD6Ls* that showed significant expression levels in fruits.

For the orange *SWEET* transporter family (Fig. 6d), Cs2g28270 and orange1.1t02626 were preferentially expressed in flowers. Cs2g28300, Cs3g14550, Cs7g02970 and orange1.1t02627 were highly expressed in fruits. Cs2g04140 had the highest expression level in leaves, and Cs3g20720 had the highest in flowers. The Cs3g14500 transcripts were abundant in leaves and fruits. Cs5g01880 was highly expressed in calluses and fruits. Cs9g04180 was strongly detected in leaves and flowers. Cs5g13290 was detected in all tested tissues. Cs5g05280, Cs5g26260, Cs5g32520 and Cs9g18460 were weakly detected in all tested tissues.

Expression profiling during orange fruit development

To gain more clues to understand the potential roles of sugar transporters, we selected a series of candidate genes and then employed a QRT-PCR approach to detect their transcriptional changes during fruit development. The

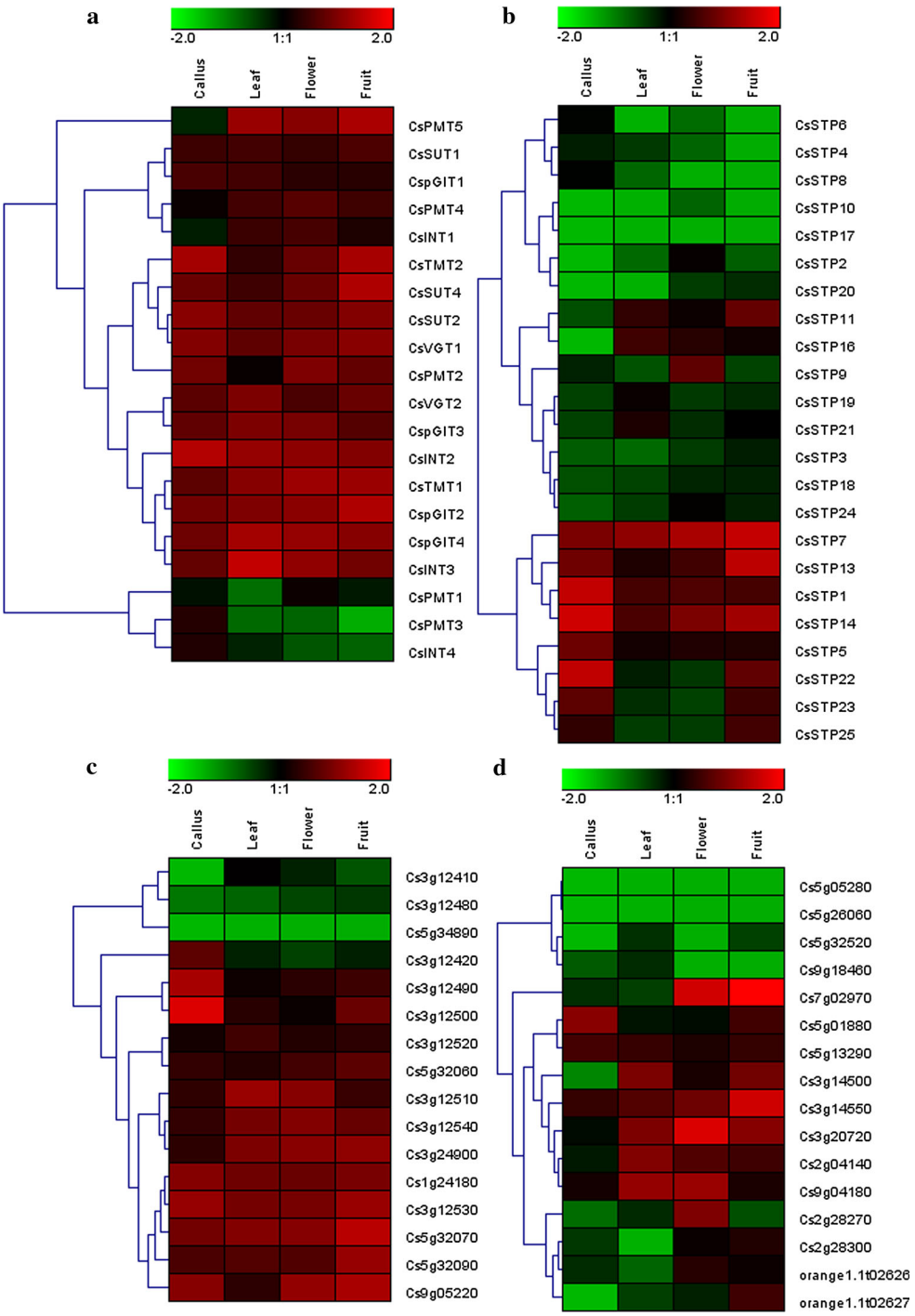

Fig. 6 Expression patterns of genes encoding *C. sinensis* *CsSUT*s, *CsSTP*s, *CsTMT*s, *CsVGT*s, *CsPMT*s, *CsINT*s, *CspGlcT*s, *CsERD6L*s and *CsSWEET*s among leaves, flowers, calluses and fruits. (**a**) *CsSUT*s, *CsTMT*s, *CsVGT*s, *CsPMT*s, *CsINT*s and *CspGlcT*s, (**b**) *CsSTP*s, (**c**) *CsERD6L*s and (**d**) *CsSWEET*s. The normalized values of the expression levels (RPKM) were retrieved from the RNA-sequencing experiment and then converted into Log10 expression values (Xu et al. 2013)

results were shown in Fig. 7. For the *CsSUT* family (Fig. 7), *CsSUT1* was lower at 120DAF than other stages, and after 150DAF, *CsSUT1* was slightly decreased as fruit ripening. The *CsSUT2* transcripts were increased from 120DAF to 180DAF, and showed a decline towards fruit ripening. The expression of *CsSUT4* displayed a continuously increase towards fruit maturation. For the *CsTMT* subfamily, *CsTMT1* presented the highest expression level at 150DAF. *CsTMT2* markedly increased as fruit ripening. For the *CsVGT* subfamily, *CsVGT1* and *CsVGT2* both significantly increased from 120DAF to 180DAF, then, *CsVGT2* was down-regulated. For the *CsPMT* subfamily, *CsPMT2*, *4* and *5* were up-regulated from 120DAF to 180DAF, then decreased. For the *CspGlcT* subfamily, *CspGlcT2* and *CspGlcT4* were up-regulated as fruit ripening, and *CspGlcT4* had a little decline at 210DAF.

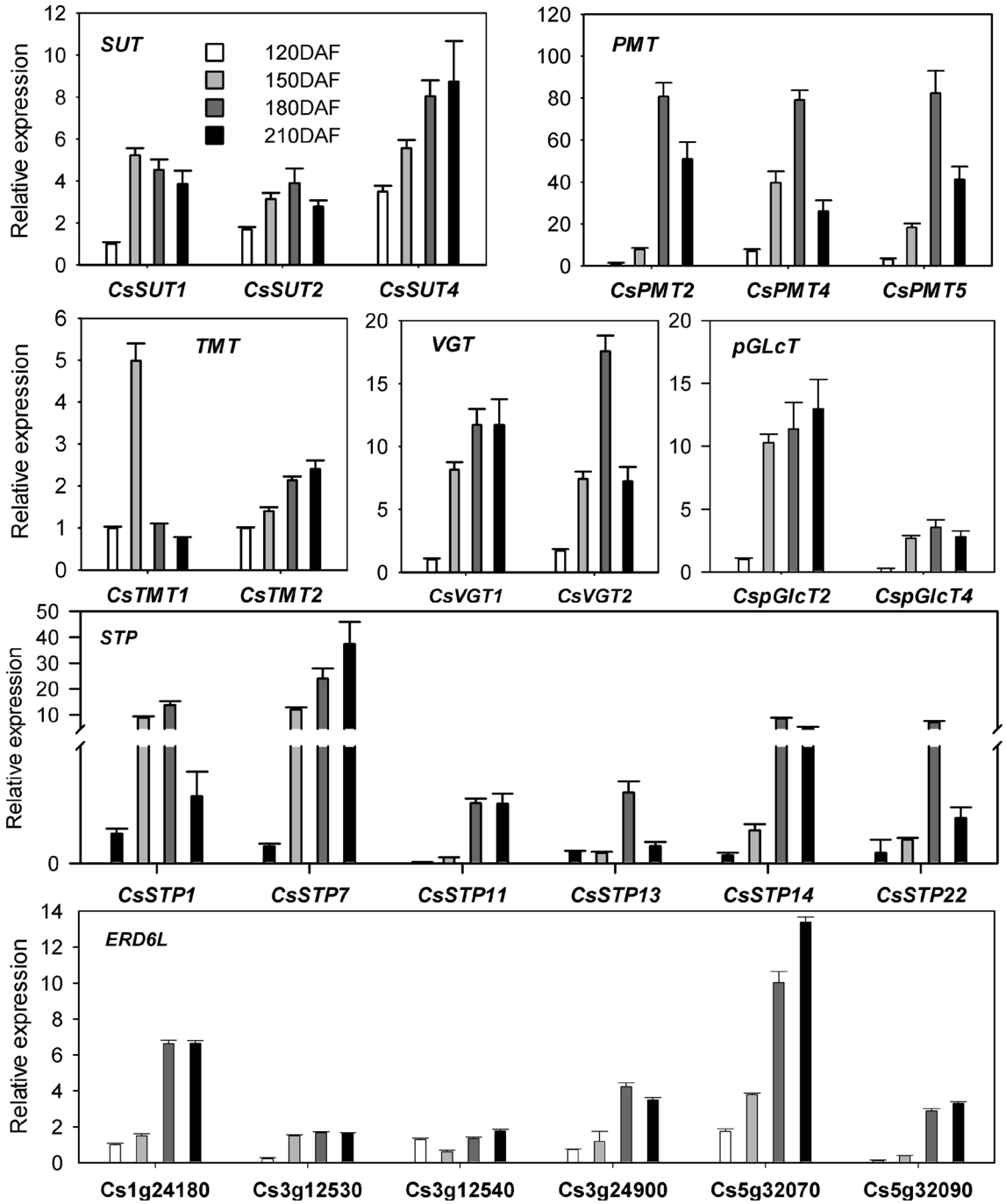

Fig. 7 Expression patterns of the selected *CsSUT*s, *CsTMT*s, *CsVGT*s, *CsPMT*s, *CspGlcT*s, *CsSTP*s and *CsERD6L*s during orange fruit development. The four developmental stages were 120, 150, 180 and 210DAF (days after flowering). The relative expression values were mean ± standard error of four replicates. *Orange Actin* was used as a reference gene. For each family, the expression level of the first member in each figure at 120DAF was set to '1'

For the *CsSTP* subfamily, 9 of the detected 23 *STP*s were highly expressed in fruits. We then selected the six ones, which had the highest expression levels in fruits, to evaluate their transcription levels as fruit ripening (Fig. 7). *CsSTP7* was obviously increased as the fruit ripened. *CsSTP1*, *CsSTP14* and *CsSTP22* were significantly increased from 120DAF to 180DAF and then decreased as the fruit reached maturation. *CsSTP11* was up-regulated from 120DAF to 180DAF and then remained unchanged. *CsSTP13* had the highest expression level at 180DAF.

For the *CsERDL6* subfamily, 12 of 16 *ERDL6* members were highly expressed in fruits. We selected the six ones that had the highest expression levels in fruits to detect their transcription levels as fruit ripening (Fig. 7). Cs5g32070 was significantly increased as fruit ripening, and as the main fruit-expressed member of the orange *ERDL6* subfamily. Cs1g24180 and Cs5g32090 were up-regulated from 120DAF to 180DAF and then remained almost unchanged. Cs3g12530 was very low at 120DAF, and then remained unchanged from 150DAF to 210DAF. Cs3g24900 presented an increase from 120DAF to 180DAF, and then decreased towards fruit ripening. Cs3g12540 showed a low level at 150DAF, and a little increase at 210DAF.

Putatively transcriptional regulation of *cis*-elements in promoters

To investigate the putative regulatory patterns in the transcriptional level, we selected 18 genes that were highly expressed in fruits and then downloaded the 2000 bp upstream promoter sequences of the start codons, except *CsVGT1,* for which an 878 bp promoter sequence was obtained. The *cis*-elements in the upstream sequences of the 18 genes were searched using the Plant Cis acting regulatory DNA Elements database. As shown in Table 1, a large number of *cis*-elements were found to be responsive to phytohormones. For example, there were on average 12–21 copies of ARR1AT, WRKY71OS and MYCCONSENSUSAT elements, which were responsive to cytokinin, gibberellin and abscisic acid, respectively. The auxin and ethylene-responsive elements, such as NTBBF1ARROLB and ERELEE4, respectively, were also identified. Additionally, there were a large number of *cis*-elements responsive to environmental factors and stresses, such as low temperature (CBFHV and IBOXCORE), light (GT1CONSENSUS and INRNTPSADB), dehydration and salicylic acid (ACGTATERD1 and WBOXATNPR1, respectively). We also found the *cis*-element SURECOREATSULTR11 was responsive to the mineral elements sulfur. Additionally, there were many *cis*-elements that were sugar-responsive, including WBOXHVISO1, SP8BF1BSP8B1B, SUCROSE BOX 3, AMYBOX1 and CGACGOSAMY3.

Discussion

Sugar transporter genes in the orange genome

In this study, we identified 77 genes encoding putative sugar transporters in the sweet orange genome. The

Table 1 Putative *cis*-elements located in the promoter regions of the orange sugar transporter genes

Name	Sequence	Response	Maximum copies/promoter	Mean copies/promoter
ARR1AT	NGATT	Cytokinin	26	21.2
NTBBF1ARROLB	ACTTTA	Auxin	4	1. 7
DPBFCOREDCDC3	ACACNNG	Abscisic acid	5	2.5
MYCCONSENSUSAT	CANNTG	Abscisic acid	24	14.3
GAREAT	TAACAAR	Gibberellin	4	1.7
WRKY71OS	TGACY	Gibberellin	19	12.6
ERELEE4	AWTTCAAA	Ethylene	3	0.9
CBFHV	RYCGAC	Low temperature	6	1.7
IBOXCORE	GATAA	Low temperature	12	7.2
GT1CONSENSUS	GRWAAW	Light	40	21.6
INRNTPSADB	YTCANTYY	Light	11	4.2
EECCRCAH1	GANTTNC	Low-CO_2	9	3.6
WBOXNTERF3	TGACY	Wounding	11	6.4
CURECORECR	GTAC	Copper, oxygen	16	6.5
SURECOREATSULTR11	GAGAC	Sulfur	5	2.0
ACGTATERD1	ACGT	Dehydration	18	9.0
WBOXATNPR1	TTGAC	Salicylic acid	7	4.4
AMYBOX1	TAACAAA	Sugar repress	5	1.2
CGACGOSAMY3	CGACG	Sugar repress	4	1.1
SP8BF1BSP8B1B	TACTATT	Sucrose induce	3	0.3
SUCROSE BOX 3	AAATCA…AA	Sucrose induce	8	4.8
WBOXHVISO1	TGACT	Sugar induce	8	3.9

Cis-elements in the promoter regions were predicted by the Plant Cis acting regulatory DNA Elements database (http://www.dna.affrc.go.jp/PLACE/index.html). The orange sugar transporter genes contained three *SUT*s, six *STP*s, two *TMT*s, two *VGT*s, two *pGlcT*s and three *PMT*s. The 2000 bp sequences upstream of the start codon were downloaded from the sweet orange genome database (http://citrus.hzau.edu.cn/orange/), except *CsVGT1*, which had an identified 878 bp upstream sequence

phylogenetic analysis showed that the SUT family, including three members located in the clades SUT1, 2 and 4, was distinctly separated from the MST family (Fig. 2a and S2). The 58 orange MSTs were divided into seven subfamilies. The *CsSTP* and *CsERD6L* subfamilies were the two largest ones, with 25 and 16 members, respectively. The distribution among the chromosomes showed that most of the *CsSTP* and *CsERD6L* members were located in the tandem repeat regions (Fig. S1). CsSTP11, 12 and 15–24, AtSTP3 and grape VvHT4 formed a separate group (Fig. 3a). The orthologous relationship of CsSTP11, 12, 15–24 and AtSTP3, as well as the adjacent genes, both reflected the collinearity of chromosome segments of orange chromosome 9 and *Arabidopsis* chromosome 5 (Fig. 4). Furthermore, it could be speculated that the tandem duplication event of orange *STP* occurred after the species separation of *Arabidopsis* and orange. Similarly, in the ERD6L subfamily, the tandem-duplicated genes were located in different clades (Fig. 3b), which indicated that the tandem duplication resulting in gene family expansion occurred after the three species separation.

Why did such gene expansion widely exist in the *STP* and *ERD6L* subfamilies? One possible explanation was that the demand for adapting to stresses or different environments led to the gene expansion. For example, under a glucose-limited environment, multiple tandem duplications of the hexose transporters *HXT6* and *HXT7* emerged in yeast (Brown et al. 1998). The yeast *HXT* gene expansion was later reported to have a positive correlation with aerobic fermentation (Lin and Li 2011). In plants, the *ERD6L* subfamily itself was named by screening putative genes responsive to dehydration or salinity stress, and was later reported to be regulated by heat, cold, drought, high salinity and wound stresses (Kiyosue et al. 1998; Poschet et al. 2011; Yamada et al. 2010). Similarly, the *STP* subfamily was regulated by biotrophic fungal infection, high salinity, programmed cell death and wound stresses (Hayes et al. 2010; Nørholm et al. 2006; Yamada et al. 2011). In our unpublished data, we also found that some *CsSTP*s were significantly induced by abscisic acid or cold treatments, and some *CsERD6L*s were induced by 4–25 folds from the microarray analysis about the genes expression patterns under sugar starvation. These findings suggested that the gene expansion in the *STP* and *ERD6L* subfamilies might reflect the demand for carbohydrates transport in stress adaption.

Potential roles of the orange sugar transporters

The expression patterns of *CsSUT1* and *CsSUT2* indicated their potential roles in both source and sink tissues. Clade SUT1 was well demonstrated to be located at the plasma membrane and to be responsible for phloem loading in companion cells, sucrose uptake into sink tissues and sucrose retrieval from apoplasmic space (Ayre 2011). Although a sink-specific expression pattern was shown by driving β-glucuronidase using the *CsSUT1* promoter in *Arabidopsis* (Singer and Cox 2012), the transcripts of *CsSUT1* were detected in leaves, flowers, fruits and calluses (Fig. 6a), which was in agreement with the previous results (Li et al. 2003). In addition, *CsSUT1* had the highest expression level at 150 DAF and slightly decreased towards fruit ripening (Fig. 7). Thus, these results indicated that *CsSUT1* might function in phloem loading in source tissues and sucrose uptake into fruit cells, especially at the early stage of fruit development. *CsSUT2* transcripts were also detected in leaves, flowers, fruits and calluses, and increased towards fruit ripening (Figs. 6a and 7). Although the SUT2 member (tomato) was proven to show no transport activity (Barker et al. 2000), other SUT2 members (AtSUC3 and PmSUC3) did display sucrose transport activity (Barth et al. 2003; Meyer et al. 2000). Furthermore, the antisense inhibition of *LeSUT2* impaired tomato fruit and seed development (Hackel et al. 2006). Therefore, CsSUT2 might play a role in sucrose uptake into fruit cells, and its transport activity needed to be confirmed.

Currently, SUT4 members were characterized as low-affinity/high-capacity sucrose/proton symporters (Ayre 2011; Kuhn and Grof 2010), and they were located in the tonoplast (Eom et al. 2011; Payyavula et al. 2011; Schneider et al. 2012). Furthermore, the vacuole was the largest subcellular organelle for carbohydrates storage, and most sucrose was mainly located in the vacuole of sink cells (Echeverria and Valich 1988; Poschet et al. 2011). Therefore, the tonoplast-located SUT4 members were believed to play important roles in regulating vacuole sucrose accumulation. Over the past years, a series of reports have demonstrated that SUT4 members played a role in sucrose distribution between vacuoles and the cytosol, and thus the regulation of SUT4 activity could affect sucrose accumulation. For example, over-expressing *AtSUC4* reduced the sucrose content in *Arabidopsis* leaves by 30 % (Schneider et al. 2012); the over-expression of *NtSUT4* in tobacco BY-2 cells induced more sucrose transportation from vacuoles to the cytosol and promoted cellulose transient accumulation during miniprotoplast culture (Okubo-Kurihara et al. 2011). In contrast, the RNAi-suppressed or T-DNA inserted mutation of *SUT4* members *PtaSUT4* and *OsSUT2* impaired sucrose transportation from vacuoles to the cytosol and resulted in the accumulation of excess sucrose in source leaves (Eom et al. 2011; Payyavula et al. 2011). Recently, the over-expression or inhibition of *FaSUT1*, which belonged to the SUT4 clade, resulted in notable changes in the sucrose content in strawberry fruits (Jia et al. 2013). These studies demonstrated that *SUT4* members played important roles in

sucrose accumulation in source or sink tissues. In orange, the transcripts of *CsSUT4* showed relatively high level in fruits (Fig. 6a) and significantly increased during fruit ripening (Fig. 7), which was accompanied by the rapid sucrose accumulation in fruits towards maturation (Fig. 1). Taken together, these findings suggested that *CsSUT4* might play important roles in orange fruit sucrose accumulation.

At least nine orange *STP*s had considerable expression levels in fruits, with most displaying up-regulated expression profiles (Fig. 6b and 7), which indicated that they were responsible for fruit sugar accumulation. For example, CsSTP13, AtSTP13 and VvHT5 were located in a single group (Fig. 3a). AtSTP13 and VvHT5 were both identified to transport glucose and fructose (Hayes et al. 2007; Nørholm et al. 2006). Moreover, over-expressing *AtSTP13* in *Arabidopsis* seedlings promoted glucose uptake (Schofield et al. 2009), and *AtSTP13* mutants showed reduced fructose and glucose uptake (Yamada et al. 2011). Later, LeHT2, which as grouped with AtSTP13 and VvHT5, was also shown to be a functional glucose and fructose transporter, and the inhibition of *LeHT2* expression in tomato fruits contributed to decrease fruit hexoses accumulation (McCurdy et al. 2010). The high expression in fruits and up-regulated pattern during fruit development suggested that *CsSTP13* played roles in importing hexoses into orange fruit juice sacs (Figs. 6b, 7).

Because the vacuole acted as the main subcellular organelle for carbohydrates storage, the tonoplast sugar transporters were gained much more attentions. *Arabidopsis* TMTs and VGTs were located in the tonoplast and functioned as hexoses importers, as well as sucrose importers for *Arabidopsis* TMTs (Aluri and Buttner 2007; Cho et al. 2010; Schulz et al. 2011; Wingenter et al. 2010; Wormit et al. 2006). Enhancing the *TMT*s expression might increase vacuole sugar accumulation. *CsTMT1* and *CsTMT2* both showed relatively high expression levels in fruits (Fig. 6a), and had the most abundant transcripts in the early and late stages of fruit development, respectively (Fig. 7). Therefore, these findings suggested that *CsTMT1* might mainly play its roles in the early stage of fruit development, and that *CsTMT2* functioned in vacuole sugar accumulation towards fruit ripening. Similarly, the expression patterns of *CsVGT1* and *CsVGT2* revealed that they played roles as fruit ripening (Fig. 6a, 7). In addition, the *Arabidopsis* ERD6L members ESL1 and AtERDL6 were targeted to the tonoplast, and characterized as glucose facilitated transporter or glucose exporter, respectively (Poschet et al. 2011; Yamada et al. 2010). We found that most of the orange *ERDL6* (12/16) members were highly expressed in fruits, and were up-regulated during fruit ripening (Fig. 6c, 7). It indicated that several orange ERDL6 members played roles in mediating fruit vacuole glucose efflux. Furthermore, two *Arabidopsis* SWEET transporter Clade IV members SWEET16 and 17 were characterized as novel tonoplast sugar transporters (Chardon et al. 2013; Guo et al. 2013; Klemens et al. 2013). We also found that the orange Clade IV members of the SWEET transporter family Cs3g14500 and Cs3g14550 showed significant expression in fruits (Fig. 6d), which indicated that they participated in sugar transport between vacuoles and the cytosol.

Although orange fruits possessed a mass of fruit-expressed and up-regulated MSTs, the fruit glucose and fructose content showed almost unchanged patterns compared with sucrose. We supposed the possibilities for this phenomenon. First, the up-regulated expression patterns of the plasma membrane-located *STP*s indicated that they enhanced apoplasmic monosaccharides transportation into the cytosol. During fruit ripening, sucrose re-synthesis via sucrose synthase and sucrose phosphate synthase activities was occurring, which needed monosaccharides as precursors (Komatsu et al. 2002; Katz et al. 2011). These imported monosaccharides may be used for sucrose re-synthesis. Glucose and fructose, therefore, did not accumulate in the ripened fruit. Second, during fruit ripening, metabolic activity such as carotenoids formation needed soluble sugars as substrates and energy sources (Xu et al. 2009). The vacuole acted as a sugar pool to supply monosaccharides via the large number of expressed tonoplast sugar transporters from the *ERDL6* and *SWEET* families. Therefore, under these multiple effects, fruit glucose and fructose could not accumulate.

In summary, our study revealed that orange had 77 sugar transporters that participated in sugar transportation from the source leaf to the fruit via the phloem, and in fruit juice sacs cells. Phylogenetic comparison suggested that the sugar transporters were highly conserved among *Arabidopsis*, grape and orange. The gene expansion in the *CsSTP* and *CsERD6L* subfamilies occurred after species separation. During fruit development, sucrose continuously accumulated as the major soluble sugar, and the glucose and fructose content remained almost unchanged. The three SUTs were expressed in leaves, flowers, calluses and fruits, and up-regulated during fruit development, indicating that they played important roles in fruit sucrose accumulation. A large number of MSTs and SWEET transporters were expressed in fruits and showed up-regulated expression patterns. However, fruit glucose and fructose did not obviously accumulate. These findings suggested that fruit sugar accumulation depended not only on transport, but also other processes such as sugar metabolism and storage. *In silico* analysis allowed us to identify many *cis*-elements responsive to sugars, phytohormones and environmental factors, which provided clues for better understanding the regulation of sugar transporter genes expression. Overall,

this study provided a comprehensive description of the orange sugar transporters and may help us to understand the sugar transportation and regulatory mechanisms during orange fruit development and maturation, as well as identify potential key genes that controlled fruit sugar accumulation.

Acknowledgments This research was supported by the Ministry of Science and Technology of China (2011CB100600) and the National Natural Science Foundation of China (31330066 and 31221062).

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